Improved Speed, Specificity, and Limit of Determination of an Aqueous Acid Extraction Thiobarbituric Acid–C_{18} Method for Measuring Lipid Peroxidation in Beef

Sri Raharjo, John N. Sofos,* and Glenn R. Schmidt

Departments of Animal Sciences and Food Science and Human Nutrition, Colorado State University, Fort Collins, Colorado 80523

The objectives of this study were to improve the speed, specificity, and limit of determination of the aqueous acid extraction thiobarbituric acid (TBA) method for measuring malonaldehyde (MA) as a marker of lipid peroxidation in ground beef. The TBA reaction time was reduced from 30 to 5 min at 94 ± 1 °C by increasing the concentration of TBA from 20 to 80 mM. The TBA-reactive substances (TBARS) in ground beef extracts and MA standard solution were stable for up to 2 and 12 days, respectively, in 5% (w/v) trichloroacetic acid (TCA) at 4 °C. Neither the MA standard nor the TBARS were stable at 35 °C in 5% (w/v) TCA. Substances from meat samples interfering with the MA–TBA reaction were removed by the use of a Sep-Pak C_{18} cartridge. The limit of determination of this modified TBA–C_{18} method was 25 times lower compared to that of the unmodified aqueous acid extraction TBA method.

INTRODUCTION

Monitoring and control of lipid peroxidation during meat processing or storage of finished products are increasingly important due to increased demand for precooked convenient meat products for home, fast-food, and institutional uses. The thiobarbituric acid (TBA) method, with its different variations, is the most widely used test for measuring the extent of lipid peroxidation in muscle foods (Gray, 1978; Melton, 1983). The basic principle of the method is the reaction of malonaldehyde and TBA to form a red malonaldehyde–TBA complex (Sinnhuber and Yu, 1958), which can be quantitated spectrophotometrically. The TBA method can be performed by (a) extracting the malonaldehyde from meat by distillation (Tarladgis et al., 1960; Rhee, 1978; Ke et al., 1984), (b) directly heating the food sample with TBA followed by separation of the red pigment produced, (c) reacting the extracted lipid portion with TBA (Pikul et al., 1983, 1989), and (d) extracting the malonaldehyde with aqueous acidic solutions followed by reaction with TBA (Witte et al., 1970; Salih et al., 1987).

Although the distillation TBA method is the most frequently used procedure for measuring lipid peroxidation in meat, and by many is regarded as the standard method for TBA analysis, it is more cumbersome and requires a longer time than the aqueous acid extraction TBA method. The aqueous acid extraction TBA method is also preferred by some workers because of its simplicity and because its results are highly correlated with those of the distillation TBA method (Pikul et al., 1989) and with sensory evaluation scores (Salih et al., 1987). In general, however, all versions of the TBA method have been criticized as being nonspecific and insensitive for the detection of low levels of malonaldehyde (Hackett et al., 1988). Other TBA-reactive substances (TBARS) including sugars and other aldehydes could interfere with the malonaldehyde–TBA reaction (Marcuse and Johansson, 1973). Therefore, a faster, more sensitive, and specific TBA method should be helpful to quality control personnel in measuring the extent of lipid peroxidation in a large number of samples in a short period of time. The objectives of this study were to increase the speed and to improve the specificity and limit of determination of the aqueous acid extraction TBA method for measuring malonaldehyde as a marker of lipid peroxidation in ground beef.

* Author to whom correspondence should be addressed.

MATERIALS AND METHODS

Reaction Time of Malonaldehyde Standard and TBARS. Malonaldehyde solutions (4 and 6 μM) were prepared by dissolving 1,1,3,3-tetraethoxypropane (Sigma Chemical Co., St. Louis, MO) into a 5% (w/v) aqueous solution of trichloroacetic acid (TCA) (Mallinckrodt, Paris, KY). Thiobarbituric acid (Sigma) solutions (20, 40, 60, 80, and 90 mM) were prepared in distilled–deionized water. Two milliliters of the TBA solutions and 2 mL of malonaldehyde solutions were reacted in a water bath (National Appliance Co., Portland, OR) of 94 ± 1 °C. The test tubes were removed from the water bath after 2, 5, 10, 15, 20, and 30 min of heating, except for the reactions of 80 and 90 mM TBA which were removed after 1, 3, 5, 7, 10, and 15 min of heating. The absorbance of the red pigment formed was scanned from 400 to 600 nm at 5-nm intervals using a spectrophotometer (Bausch and Lomb Inc., Rochester, NY).

An experiment similar to the above was performed with raw and cooked ground beef replacing the pure malonaldehyde. Ten grams of raw (18–22% fat) or cooked (12–15% fat) ground beef samples, after 0 or 24 h of aerobic storage at 4 °C, were homogenized with 40 mL of 5% (v/v) aqueous TCA solution in an Osterizer blender (Sunbeam Corp., Milwaukee, WI) for 1 min. Butylated hydroxytoluene (BHT) (Sigma) was added prior to homogenization at a level of 0.15% based on lipid content (Pikul et al., 1989). The meat slurry was centrifuged (Beckman Instruments Inc., Fullerton, CA) at 10000*g* for 5 min. The supernatant was recovered and filtered through a Whatman microfiber glass filter grade C (Whatman, Hillsboro, OR) into a 50-mL volumetric flask. The volume of the filtrate was adjusted to 50 mL with 5% (w/v) TCA. A 2-mL portion of the filtrate was reacted with 2 mL of either 20 mM TBA for 0, 5, 10, 15, 20, 30, and 40 min or 80 mM TBA for 0, 2, 5, 10, 15, 20, and 30 min under the conditions described above. The absorbance of the red pigment was scanned as described above.

Stability of Malonaldehyde Standard and TBARS. Raw and cooked meat extracts (50 mL each) containing TBARS and 4 μM malonaldehyde in 5% (w/v) aqueous TCA solution (50 mL) were prepared as described above. One portion (25 mL) of each of these extracts in Erlenmeyer flasks was wrapped with aluminum foil and stored at 4 °C, while the other portion (25

0021-8561/92/1440-2182$03.00/0 © 1992 American Chemical Society

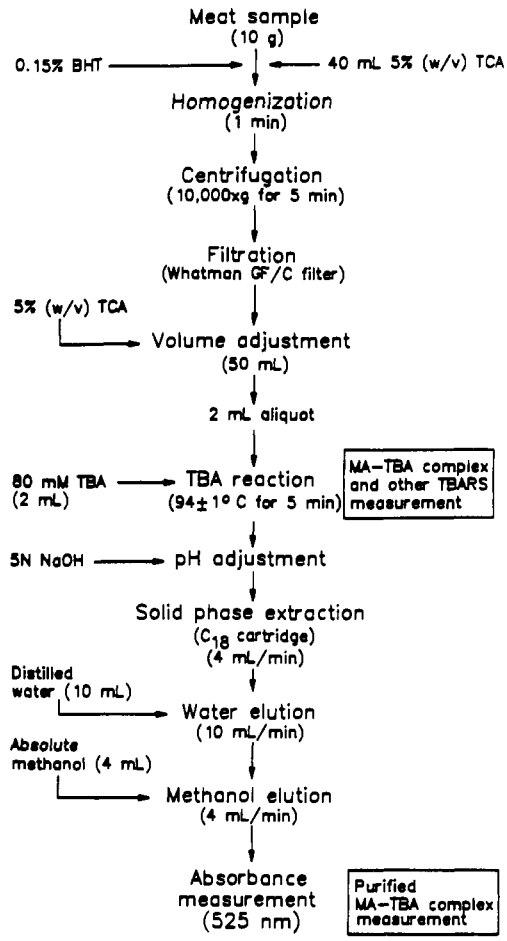

Figure 1. Diagram indicating the steps involved in the aqueous acid extraction thiobarbituric acid–C_{18} (TBA–C_{18}) method. BHT, butylated hydroxytoluene; TCA, trichloroacetic acid; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; MA, malonaldehyde.

mL) was wrapped and stored at 35 °C. Samples (2 mL) from each of these portions, after storage for 0, 1, 2, 4, 8, and 12 days, were reacted with 2 mL of 80 mM TBA under the conditions described above, except that the heating time was 5 min. The absorbance of the red pigment was measured at 525 nm.

Aqueous Acid Extraction TBA–C_{18} Method. The TBA–C_{18} method was performed as described in Figure 1. The pH of the red pigment formed was adjusted to approximately 1, 2, 4, 5.5, 6.5, 7.5, 8.5, 10, and 12 using 5 N NaOH (Mallinckrodt) and 0.2 mL of 3% (w/v) phosphate buffer of pH 7.2 (Becton Dickinson and Co., Cockeysville, MD) prior to loading into a solid-phase extraction (Sep-Pak) C_{18} cartridge (Waters, Milford, MA). Prior to use, the cartridge was washed with 10 mL of absolute methanol (Mallinckrodt) followed by 10 mL of distilled water, each at a flow rate of approximately 20 mL/min. The malonaldehyde–TBA complex was recovered from other TBARS complexes by eluting the C_{18} cartridge with absolute methanol, and the absorbance of the methanol eluent was measured spectrophotometrically at 525 nm. The other TBARS complexes left in the cartridge were considered as interfering substances.

Extraction Recovery and Limit of Determination. Extraction recoveries of malonaldehyde and limits of determination for the aqueous acid extraction TBA–C_{18} and the unmodified aqueous acid extraction TBA methods were determined using pre-extracted raw or cooked ground beef residues. The pre-extracted meat residues were prepared by homogenizing the meat (10 g) with 40 mL of 5% (w/v) TCA in an Osterizer blender for 1 min, followed by centrifugation and filtration as described above. The supernatant was discarded; the meat residue was reused and subjected to the same extraction step for three additional times. The solid materials resulting from these four consecutive extractions were pooled and designated pre-extracted ground beef residue which should technically be "free" of malonaldehyde.

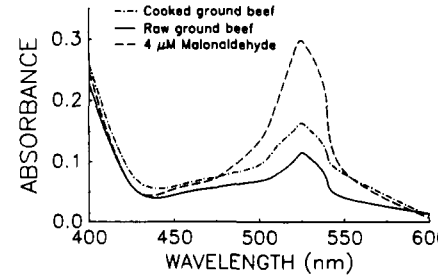

Figure 2. Absorbance scans of red pigment produced by the reaction of thiobarbituric acid (TBA) with pure malonaldehyde (4 μM) in 5% (w/v) trichloroacetic acid (TCA) and with TBA-reactive substances in aqueous solid extracts of raw and cooked ground beef.

Several graded levels of pure malonaldehyde (0, 50, 100, 200, 300, and 400 nmol) solutions were added to 10-g portions of the pre-extracted ground beef residues and subsequently analyzed by the unmodified aqueous acid extraction TBA method as described by Salih et al. (1987), except that 5% (w/v) TCA was used as the extracting medium instead of 4% perchloric acid, which is known as a strong oxidizing agent. Another set of graded levels of malonaldehyde (0, 2, 4, 8, 12, and 16 nmol) was added to separate 10-g portions of the pre-extracted ground beef residues. These spiked samples were then subjected to the newly developed aqueous acid extraction TBA–C_{18} method. In this particular analysis, all of the meat extract (50 mL) was reacted with 50 mL of 80 mM TBA under the conditions described above. The red complex produced from this reaction (100 mL) was concentrated by passing the solution through a C_{18} cartridge following the procedures described in Figure 1. Standard curves were prepared by adding the graded levels of pure malonaldehyde into 50 mL of 5% (w/v) TCA followed by TBA reaction. The extraction recovery of malonaldehyde and the limit of determination of both the unmodified aqueous acid extraction TBA and the TBA–C_{18} methods were calculated following the procedures described by Thier and Zeumer (1987).

Statistical Analyses. Reaction time to produce maximum absorbance and stability of the malonaldehyde standard and TBARS in the meat extracts were analyzed by linear regression (Steel and Torrie, 1980). The compound was considered stable when the slope of the regression line was equal to zero at $\alpha = 0.05$. The capability of the Sep-Pak C_{18} cartridge for the removal of interfering substances was evaluated by analysis of variances. Each experiment in this study was replicated four times.

RESULTS AND DISCUSSION

Reaction Time of Malonaldehyde Standard and TBARS. The wavelength of maximum absorbance of the pure malonaldehyde–TBA complex and of TBARS obtained from raw or cooked ground beef samples was 525 nm (Figure 2). Other studies on the measurement of lipid peroxidation using various versions of the TBA method have reported several different wavelengths of maximum absorbance such as 530 (Tarladgis et al., 1964), 531 (Salih et al., 1987), 532 (Tomas and Funes, 1987; Pikul et al., 1989), 535 (Rhee et al., 1984), and 538 nm (Ke et al., 1984; Hoyland and Taylor, 1989). The wavelength at which the absorbance of the malonaldehyde–TBA complex is measured should be that wavelength at which a given spectrophotometer results in maximum absorbance in the 400–600-nm region.

The time needed for malonaldehyde to react with TBA to produce maximum absorbance of the red malonaldehyde–TBA complex was affected by the concentration of the TBA solution. Increasing the TBA concentration from 20 to 80 or 90 mM reduced the reaction time from 30 to approximately 5 min (data not shown). This is apparently due to more TBA being available for the reaction. Also, increasing the TBA concentration from 20 to 90 mM decreased the pH of the reaction mixtures from 2.1 to 1.4

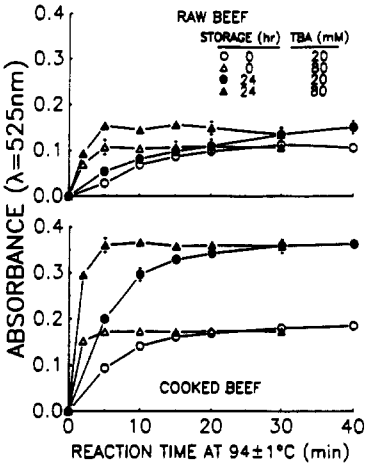

Figure 3. Reaction time of thiobarbituric acid reactive substances (TBARS) from raw and cooked ground beef extracts and 20 or 80 mM thiobarbituric acid at 94 ± 1 °C to produce maximum absorbance of red TBARS complexes. The meat samples were stored at 4 ± 1 °C for 0 or 24 h.

(data not shown). A decrease in the pH of the reaction mixture enhanced the speed of the reaction of malonaldehyde and TBA (Kwon and Watts, 1964). Increasing the malonaldehyde concentration from 4 to 6 μM did not affect the reaction time. As expected, the 6 μM malonaldehyde resulted in significantly ($P < 0.05$) higher maximum absorbance (0.44) than that of 4 μM malonaldehyde (0.30). The maximum absorbance produced was also unaffected by the TBA concentration. Increasing the TBA concentration from 80 to 90 mM did not further reduce the reaction time (data not shown). In addition, at room temperature the 90 mM TBA tended to recrystallize relatively quickly.

Similar conclusions could be reached when the 80 mM TBA was used for reaction with TBARS in raw or cooked ground beef extracts. The use of 80 mM TBA, instead of 20 mM TBA, reduced the reaction time to reach maximum absorbance from approximately 40 to approximately 5 min in raw and cooked ground beef samples (Figure 3). The most frequently used concentrations of TBA for malonaldehyde–TBA complex formation in TBA tests reported has been 20 mM (Tarladgis et al., 1960; Salih et al., 1987; Pikul et al., 1989). Since the use of the higher level (80 mM) of TBA did not interfere with the analysis, it is recommended that this concentration be used to increase the speed of the TBA test.

Stability of Malonaldehyde and TBARS. Pure malonaldehyde standard (4 μM) was stable in 5% (w/v) aqueous TCA solution for at least 12 days at 4 °C (data not shown). This is in agreement with the study by Kwon and Watts (1964), who reported that low concentrations (less than 25 μM) of malonaldehyde solution were stable under neutral and acidic conditions for 20 days at 4 °C. However, the TBARS obtained from raw or cooked ground beef samples were stable for only 2 days at 4 °C (data not shown).

At 35 °C, neither the pure malonaldehyde standard nor the TBARS extracted from ground beef were stable (data not shown). This should be due to the malonaldehyde-containing solutions becoming prone to aldol-type self-condensation, leading to formation of dimers and trimers which have reacted differently with TBA (Esterbauer et al., 1991). This is also in close agreement with the results of Gutteridge et al. (1977), who reported that malonaldehyde standard prepared under acidic conditions at 60 °C produced at least five different condensation products. Therefore, it is recommended that the aqueous acid meat extracts be stored at low temperature (4 °C) to maintain their stability before the TBA test is completed within 2 days.

Specificity of the TBA–C_{18} Method. The ability of the C_{18} cartridge to effectively separate the malonaldehyde–TBA complex from other TBARS complexes was affected by the pH of the red solutions. A successful and reproducible separation could only be achieved when the pH of the chromogen produced by the TBA reaction was adjusted in the range 5.5–8.5 (data not shown). This finding is in agreement with results of Squires (1990), who recommended adjusting the pH of the chromogen to approximately 7.0.

Pure malonaldehyde–TBA complex, prepared from malonaldehyde standard, was passed through the C_{18} cartridge to determine whether the methanol eluent contained only the malonaldehyde–TBA complex. This malonaldehyde standard did not contain any other TBARS as indicated by the fact that almost all (100%) of the pure malonaldehyde–TBA complex was recovered from the cartridge. The results (not shown) indicated, however, that approximately 12.7–16.8% of the TBARS in the raw beef extract and 27.3–29.1% of the TBARS in the cooked beef extract were not recovered by absolute methanol elution. This suggested that the raw and cooked ground beef extract contained 12.7–16.8 and 27.3–29.1% interfering substances, respectively.

Under strongly acidic or basic conditions, separation of the malonaldehyde–TBA complex from other TBARS in the C_{18} cartridge was unsuccessful. At pH 10 or higher the separation failed because a major portion (approximately 80%) of the red TBARS complexes was leaking out from the cartridge when the sample was loaded. At pH 4.0 or lower, all of the red TBARS complexes were retained in the cartridge as indicated by relatively small leakage (less than 4%) of the TBARS complexes. However, the separation was unsatisfactory because the amounts of malonaldehyde–TBA complex recovered from the cartridge were relatively low (15–35%).

Extraction Recovery and Limit of Determination. Extraction recoveries of malonaldehyde in raw and cooked ground beef determined by the unmodified aqueous acid extraction TBA method were 76 and 79%, respectively. When analyzed by the aqueous acid extraction TBA–C_{18} method, the same meat samples resulted in recovery values of 78% in raw and 80% in cooked ground beef. The extraction recovery values obtained by these two methods were not significantly ($P > 0.05$) different in raw or cooked ground beef. Studies using the aqueous acid extraction techniques have reported extraction recovery values of 57 (Siu and Draper, 1978), 62 (Newburg and Concon, 1980), 62.8 (Izumimoto et al., 1990), 93 (Salih et al., 1987), 94 (Witte et al., 1970; Pikul et al., 1989), and 94.8% (Squires, 1990). These differences could be due to different types and concentrations of acid used, type of meat and its freshness, and the procedures used for recovery determination.

The smallest level of pure malonaldehyde added (1 nmol/mL of meat extract) to the meat sample was detected by the unmodified aqueous acid extraction TBA method to be approximately 0.77 nmol/mL of meat extract. This value was significantly ($P < 0.05$) different from zero. Thus, the limit of determination of the unmodified aqueous acid extraction TBA method was 1 nmol of malonaldehyde equivalents/mL of meat extract. Since the total volume of meat extract (50 mL) was originated from 10 g of meat sample, the limit of determination mentioned above was equivalent with a TBA number of 0.36 mg of malonal-

dehyde equivalents/1000 g of meat. Since the recovery values in this experiment were in the range 76–80%, their corresponding K values were found in the range 6.0–6.3, as calculated according to the procedure of Pikul et al. (1989). Therefore, any TBA numbers lower than 0.36 obtained by their particular method should not be considered real values.

The smallest level of pure malonaldehyde added (0.04 nmol/mL of meat extract) to the meat sample was detected by the aqueous acid extraction TBA–C_{18} method to be approximately 0.03 nmol/mL of meat extract, and this value was significantly ($P < 0.05$) different from zero. Therefore, the use of a solid-phase extraction Sep-Pak C_{18} cartridge in the aqueous acid extraction TBA–C_{18} method improved the limit of determination from 1 nmol of malonaldehyde equivalents/mL of meat extract (or TBA value of 0.36) to 0.04 nmol malonaldehyde equivalents/mL of meat extract (or TBA value of 0.014). This means that the limit of determination of malonaldehyde by the aqueous acid extraction TBA–C_{18} method was 25 times lower than that by the unmodified aqueous acid extraction TBA method. This improvement of limit of determination was due to the ability of the C_{18} cartridge to concentrate the malonaldehyde–TBA complex produced after the TBA reaction.

ACKNOWLEDGMENT

This work was supported in part by the Agricultural Experiment Station of Colorado State University and by FANDARC (Food and Nutrition Development and Research Center) of Gadjah Mada University, Indonesia.

LITERATURE CITED

Esterbauer, H.; Schaur, R. J.; Zollner, H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biol. Med.* **1991**, *11*, 81–128.

Gray, J. I. Measurement of lipid oxidation. *J. Am. Oil Chem. Soc.* **1978**, *55*, 539–546.

Gutteridge, J. M. C.; Heys, A. D.; Lunec, J. Fluorescent malondialdehyde polymers from hydrolyzed 1,1,3,3-tetramethoxypropane. *Anal. Chim. Acta* **1977**, *94*, 209–211.

Hackett, C.; Linley-Adams, M.; Lloyd, B.; Walker, V. Plasma malondialdehyde: A poor measure of in vivo lipid peroxidation. *Clin. Chem.* **1988**, *34*, 208.

Hoyland, D. V.; Taylor, A. J. A modified distillation method for the detection of fat oxidation in foods. *Int. J. Food Sci. Technol.* **1989**, *24*, 153–161.

Izumimoto, M.; Kataoka, K.; Miyamoto, T. Mathematical approach for determining TBARS in meat by extraction and distillation methods. *Agric. Biol. Chem.* **1990**, *54*, 1311–1313.

Ke, P. J.; Cervantes, E.; Robles-Martinez, C. Determination of thiobarbituric acid reactive substances (TBARS) in fish tissue by an improved distillation-spectrophotometric method. *J. Sci. Food Agric.* **1984**, *35*, 1248–1254.

Kwon, T. W.; Watts, B. M. Malonaldehyde in aqueous solution and its role as a measure of lipid oxidation in foods. *J. Food Sci.* **1964**, *29*, 294–302.

Marcuse, R.; Johansson, L. Studies on the TBA test for rancidity grading: II. TBA reactivity of different aldehyde classes. *J. Am. Oil Chem. Soc.* **1973**, *50*, 387–391.

Melton, S. T. Methodology for following lipid oxidation in muscle foods. *Food Technol.* **1983**, *37* (7), 105–116.

Newburg, D. S.; Concon, J. M. Malonaldehyde concentrations in food are affected by cooking conditions. *J. Food Sci.* **1980**, *45*, 1681–1683, 1687.

Pikul, J.; Leszczynski, D. E.; Kummerow, F. A. Elimination of sample autoxidation by butylated hydroxytoluene additions before thiobarbituric acid assay for malonaldehyde in fat from chicken meat. *J. Agric. Food Chem.* **1983**, *31*, 1338–1342.

Pikul, J.; Leszczynski, D. E.; Kummerow, F. A. Evaluation of three modified TBA methods for measuring lipid oxidation in chicken meat. *J. Agric. Food Chem.* **1989**, *37*, 1309–1313.

Rhee, K. S. Minimization of further lipid peroxidation in the distillation 2-thiobarbituric acid test of fish and meat. *J. Food Sci.* **1978**, *43*, 1776–1778, 1781.

Rhee, K. S.; Dutson, T. R.; Smith, G. C. Enzymic lipid peroxidation in mirosomal fractions from beef skeletal muscle. *J. Food Sci.* **1984**, *49*, 675–679.

Salih, A. M.; Smith, D. M.; Price, J. F.; Dawson, L. E. Modified extraction 2-thiobarbituric acid method for measuring lipid oxidation in poultry. *Poult. Sci.* **1987**, *66*, 1483–1488.

Sinnhuber, R. O.; Yu, T. C. 2-thiobarbituric acid method for the measurement of rancidity in fishery products. II. The quantitative determination of malonaldehyde. *Food Technol.* **1958**, *12* (1), 9–12.

Siu, G. M.; Draper, H. H. A survey of the malonaldehyde content of retail meats and fish. *J. Food Sci.* **1978**, *43*, 1147–1149.

Squires, E. J. High performance liquid chromatographic analysis of the malondialdehyde content of chicken liver. *Poult. Sci.* **1990**, *69*, 1371–1376.

Steel, R. G. D.; Torrie, J. H. *Principles and procedures of statistics*, 2nd ed.; McGraw-Hill: New York, 1980.

Tarladgis, B. G.; Watts, B. M.; Younathan, M. T.; Dugan, L. R., Jr. A distillation method for the quantitative determination of malonaldehyde in rancid foods. *J. Am. Oil Chem. Soc.* **1960**, *37*, 44–48.

Tarladgis, B. G.; Pearson, A. M.; Dugan, L. R., Jr. Chemistry of the 2-thiobarbituric acid test for determination of oxidative rancidity in foods. II. Formation of the TBA-malonaldehyde complex without acid-heat treatment. *J. Sci. Food Agric.* **1964**, *15*, 602–607.

Thier, H. P.; Zeumer, H. *Manual of pesticide residue analysis*; VCH Publishers: New York, 1987; Vol. 1.

Tomas, M. C.; Funes, J. Application of 2-thiobarbituric acid reaction to exudates of frozen and refrigerated meats. *J. Food Sci.* **1987**, *52*, 575–579.

Witte, V. C.; Krause, G. F.; Bailey, M. E. A new extraction method for determining 2-thiobarbituric acid values of pork and beef during storage. *J. Food Sci.* **1970**, *35*, 582–585.

Received for review April 20, 1992. Revised manuscript received August 4, 1992. Accepted August 20, 1992.

Registry No. MA, 542-78-9.